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Chaperoning HMGA2 Protein Protects Stalled Replication Forks in Stem and Cancer Cells

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SUMMARY

Maintaining genome integrity requires the accurate and complete replication of chromosomal DNA. This is of the utmost importance for embryonic stem cells (ESCs), which differentiate into cells of all lineages, including germ cells. However, endogenous and exogenous factors frequently induce stalling of replication forks in every cell cycle, which can trigger mutations and chromosomal instabilities. We show here that the oncofetal, nonhistone chromatin factor HMGA2 equips cells with a highly effective first-line defense mechanism against endonucleolytic collapse of stalled forks. This fork-stabilizing function most likely employs scaffold formation at branched DNA via multiple DNA-binding domains. Moreover, HMGA2 works independently of other human factors in two heterologous cell systems to prevent DNA strand breaks. This fork chaperone function seemingly evolved to preserve ESC genome integrity. It is hijacked by tumor (stem) cells to also guard their genomes against DNA-damaging agents widely used to treat cancer patients.

INTRODUCTION

The faithful completion of chromosomal DNA replication is a key cellular process. It prevents mutations and chromosome/genome instability. This is of particular importance for pluripotent embryonic stem cells (ESCs), which differentiate into all somatic cell lineages and germ cells (Cervantes et al., 2002; Petermann and Helleday, 2010). However, a fraction of replication forks arrest during each cell cycle when they encounter obstacles such as DNA lesions or DNA secondary structures in repeat sequences present, for example, in telomeres. Replication stalling often leads to nucleolytic fork collapse (Branzei and Foiani, 2010; Mirkin and Mirkin, 2007).

Elaborate cell signaling and DNA recombination/repair pathways exist in pro- and eukaryotic cells to cope with nucleolytic lesions at arrested forks (Branzei and Foiani, 2010; Budzowska and Kanaar, 2009; Paulsen and Cimprich, 2007; Zegerman and Diffley, 2009). From a cellular perspective, however, the physical and functional maintenance of stalled forks appears to be a safer and more economical solution to a seemingly unavoidable problem. Hence, it is surprising that a more general pathway of repair-independent fork protection has only recently emerged. Its key feature is the recognition of single-stranded DNA (ssDNA) regions at stalled or regressed forks.

In *Escherichia coli*, the RecFOR proteins promote loading of RecA filaments on ssDNA. Subsequent formation of triple-stranded RecA complexes on nascent DNA is thought to physically stabilize forks and prevent strand breakage or degradation until an obstacle is removed or a second fork comes to the rescue (Courcelle et al., 2003; Courcelle and Hanawalt, 2003; Masai et al., 2010). Furthermore, it was shown that RecA alone can trigger disassembly of replisomes at stalled forks, which might contribute to fork stability and recovery (Lia et al., 2013).

In mammalian cells, a similar repair-independent protection mechanism was uncovered more recently. It involves Fanconi anemia and the tumor-suppressor BRCA1/2 proteins, which stabilize nucleoprotein filaments composed of the mammalian RecA homolog RAD51 and nascent ssDNA at stalled forks. This in turn protects DNA strands against degradation by the MRE11 nuclease and further stabilizes arrested forks until replication recovery can be achieved (Hashimoto et al., 2010; Lomonosov et al., 2003; Schlacher et al., 2011, 2012). Furthermore, in

eukaryotic cells, replication protein A (RPA) binds to ssDNA at stalled forks and triggers ataxia telangiectasia RAD3-related (ATR) ATR-ATRIP checkpoint activation. This in turn results in checkpoint kinase-1 (CHK1) activation mediated by TOPBP1, Claspin, and BRCA1 (Aressy and Greenberg, 2012).

Claspin is also an essential component of the so-called fork protection complex (FPC) made up of the Tim/Tipin and AND1 proteins. The FPC is evolutionarily conserved and is thought to be part of translocating replisomes where it physically links helicase and polymerase activities, thereby stabilizing stalled forks and facilitating successful fork restart (Errico and Costanzo, 2010, 2012; Tourrière and Pasero, 2007). The precise downstream mechanism(s) that ultimately stabilizes stalled forks is, however, still elusive and requires phosphorylation of a number of proteins within a replisome as well as a sufficient supply of RPA (Bermejo et al., 2011; Branzei and Foiani, 2010; Cimprich and Cortez, 2008; Toledo et al., 2013). Together, these repair/recombination-independent fork protection mechanisms, which usually start at ssDNA regions, reduce the occurrence of DNA strand breaks and chromosomal aberrations, and promote cell survival. Given their apparent importance, the existence of additional mechanisms, especially in stem cells, can be anticipated.

The mammalian high-mobility group AT-hook 2 (HMGA2) protein is evolutionarily highly conserved in mammals and is expressed in ESCs and during early developmental stages. HMGA2 appears to be absent from normal somatic tissues (Rogalla et al., 1996), but is reexpressed in most malignant human neoplasias due to Lin28-mediated let-7 miRNA degradation (Li et al., 2013). Moreover, the level of expression strongly correlates with the degree of malignancy and metastatic potential (Abe et al., 2003; Dröge and Davey, 2008; Fusco and Fedele, 2007; Meyer et al., 2007).

HMGA2 harbors a C-terminal acidic tail and three independent DNA-binding domains that recognize AT-rich duplex sequences via the minor groove (Cleynen and Van de Ven, 2008; Huth et al., 1997; Reeves and Nissen, 1990). In general, HMGA proteins are considered architectural factors and interact with chromatin in a highly dynamic manner (Harrer et al., 2004; Reeves and Nissen, 1990). In the context of chromatin, different DNA-binding modes have been proposed (Pfannkuche et al., 2009). For example, the three AT-hooks can bind separately to different DNA molecules in an all *trans* configuration to create a chromatin scaffold (Vogel et al., 2011). Interestingly, the HMGA1a protein exhibits higher binding affinities to Holliday junctions (HJs) than to canonical AT-rich duplex DNA, and recognizes HJs through multiple contacts at the center of DNA branch points (Hill et al., 1999; Hill and Reeves, 1997). Furthermore, a wheat HMGA homolog also binds to HJs with high affinity (Zhang et al., 2003).

In addition to the known involvement of HMGA2 in the regulation of gene expression and cell transformation/differentiation processes (Cleynen and Van de Ven, 2008; Fedele et al., 2010; Pfannkuche et al., 2009), we recently provided evidence for a role of HMGA2 (and HMGA1) in the protection of HMGA-positive cancer cells against DNA-damage-induced cytotoxicity (Summer et al., 2009). We attributed this protective effect to a dRP/AP lyase activity of HMGA2 and a link to base excision repair. Strikingly, however, the strongest protective effect was detected against hydroxyurea-induced cell death, hinting at a possible additional role of HMGA2 at stalled forks.

In this study, we find that HMGA2 is a bona fide replication fork chaperone in ESCs and cancer cells that substantially stabilizes physical fork integrity. Strikingly, in *E. coli*, human HMGA2 alone is sufficient to both fully complement the RecA protein in its known replication fork-stabilizing role and suppress antimicrobial peptide-induced cleavage specifically at branched DNA structures in vivo. In *Saccharomyces cerevisiae*, HMGA2 partially complements the fork-stabilizing function of ATR/Mec1 by reducing the occurrence of collapsed, regressed forks and their subsequent endonucleolytic collapse. We have thus uncovered an important repair/recombination-independent replication fork protection pathway that is unique to stem and cancer cells that express HMGA2.

RESULTS

HMGA2 Stably Associates with Sites of DNA Replication

Detection of endogenous HMGA2 in human fibrosarcoma HT1080 cells by immunostaining revealed the expected colocalization with DAPI-stained DNA inside the cell nucleus (Figure S1A, top panels). We noted, however, the existence of numerous diffuse HMGA2 foci and hypothesized that they represent a distinct, more stably associated chromatin fraction of HMGA2. Next, a staining protocol allowed us to substantially reduce the weaker chromatin-associated HMGA2 fraction and to observe clear, individual HMGA2 foci (Figure S1A, bottom panels).

We pulse-labeled DNA in human fibrosarcoma HT1080 and mouse ESCs (mESCs) with 5-iodo-2′deoxyuridine (IdU) and detected more than 80% colocalization of IdU with HMGA2 signals. This was the case in both the absence and presence of hydroxyurea (HU) (Figures 1A and 1E). HU causes arrest of replication forks through the inhibition of ribonucleoside diphosphate reductase (Krakoff et al., 1968). This result suggested that a fraction of HMGA2 molecules are stably associated with both ongoing and stalled replication sites.

We next determined whether HMGA2 colocalized with RPA and/or proliferating cell nuclear antigen (PCNA) before and after HU treatment. RPA and PCNA are bound at ongoing and arrested forks (Fanning et al., 2006; Sirbu et al., 2011). The vast majority of cells exhibited costaining for HMGA2/RPA and HMGA2/PCNA, and quantification per cell revealed that more than 80% of RPA signals and up to 90% of PCNA signals colocalized with distinct HMGA2 foci irrespective of HU treatment (Figures 1B and 1F, and 1C and 1G, respectively). Similar results were obtained with a different primary HMGA2 antibody for RPA costaining (Figure S1B). As expected, quantification of immunostaining between RPA and PCNA also revealed substantial (>90%) colocalization before and after HU treatment (Figures 1D and 1H).

To provide further evidence that HMGA2 is localized at replication sites containing ongoing and/or stalled replication forks, we performed proximity ligation assays (PLAs), which are a sensitive measure that can detect in situ whether two proteins are colocalized and not more than 40 nm apart (Söderberg et al., 2006). We counted PLA foci in at least 55 cell nuclei per sample, and the data revealed colocalization for HMGA2 and RPA, HMGA2 and

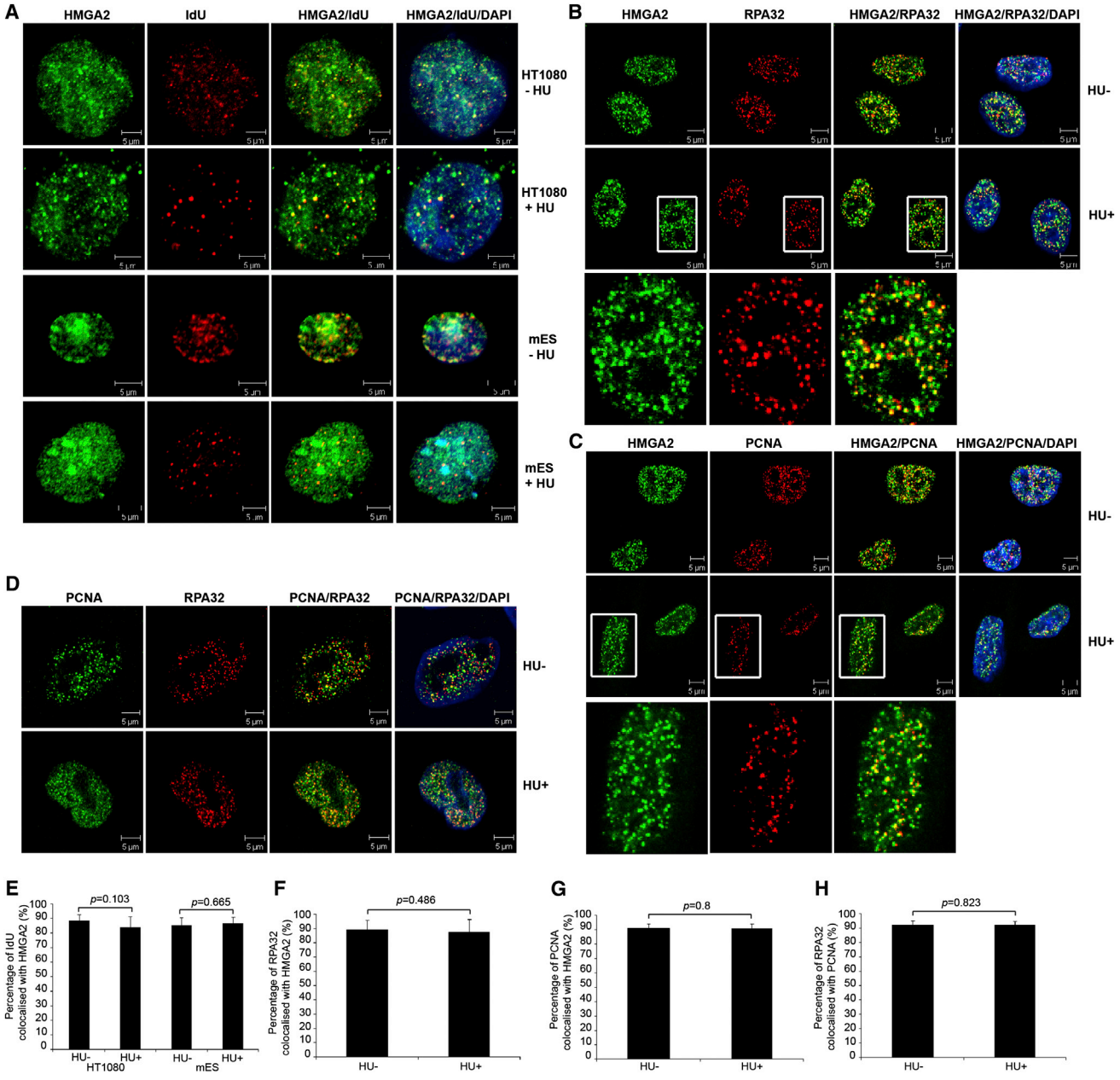

Figure 1. HMGA2 Associates with Replication Sites Containing Ongoing and Stalled Forks

(A) Human HT1080 fibrosarcoma cells and mESCs were pulse labeled for 25 min with IdU and treated for 24 hr with HU (+HU) followed by immunostaining with antibodies recognizing HMGA2 (green) and IdU (red). Untreated (−HU) cells were processed for immunostaining immediately after IdU labeling. Genomic DNA was visualized by DAPI staining.

(B) Replication foci associated with HMGA2 were identified by coimmunostaining for RPA and HMGA2. See also Figure S1B.

(C) Coimmunostaining between HMGA2 and PCNA.

(D) Coimmunostaining between PCNA and RPA32.

(E–H) Quantification of colocalizing signals per cell as depicted in (A)–(D), respectively. For each sample, 20 cells were analyzed using LSM710 ZEN software and the mean percentage value + SD was determined. Two independent experiments were quantified, yielding very similar results. We show the results of one experiment.

PCNA, and, as a control, RPA and PCNA, irrespective of the presence or absence of HU (Figures 2A and 2B). Furthermore, the data revealed that PLA foci were significantly enriched in cells engaged in DNA replication, as evidenced by incorporation of 5-ethynyl-2′-deoxyuridine (EdU; Figure 2B, EdU− and EdU+). Controls employing only one of the four primary antibodies showed significantly reduced numbers of PLA foci per cell (Figures 2A–2C).

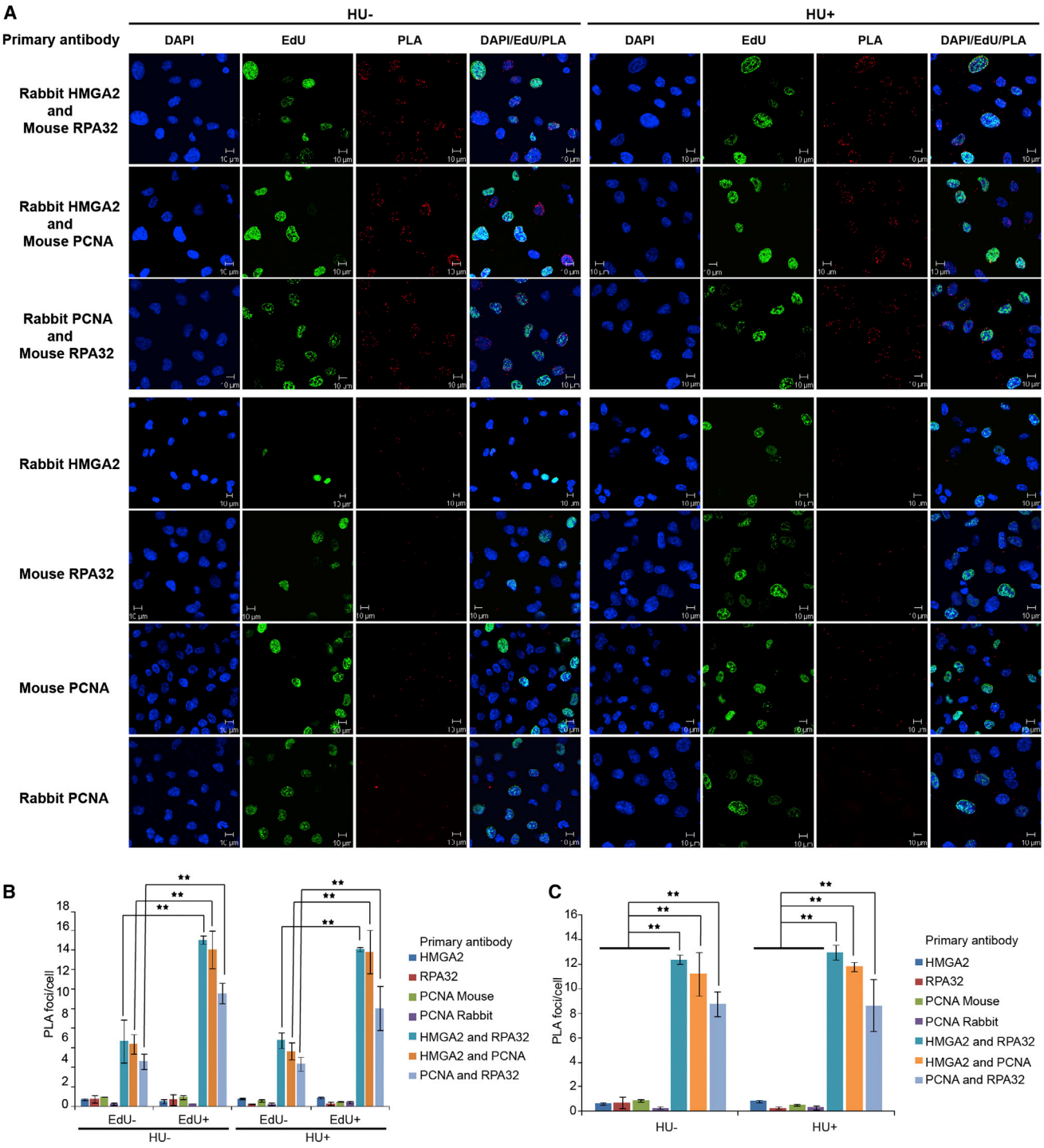

Figure 2. HMGA2 Is in Close Proximity to Replication Factors RPA32 and PCNA

(A) Human HT1080 fibrosarcoma cells were labeled for 15 min with EdU and treated with hydroxyurea (+HU) for 16 hr in the continued presence of EdU. Untreated (−HU) cells were processed for PLAs immediately after 15 min of pulse labeling. Genomic DNA was visualized by DAPI staining. The presence of primary antibodies used for PLA and controls is indicated at the left side of the panels.

(B and C) Quantification of PLA foci. For each sample, PLA foci in at least 55 cells (EdU(+) plus EdU(−)) were counted and the mean values were determined. We show mean values, SDs, and statistically significant differences from two independent experiments for each sample (**$p < 0.01$).

We also performed chromatin immunoprecipitation experiments on small chromatin fragments (0.2–2 kb) using antibodies against bromodeoxyuridine (BrdU)-labeled nascent DNA to further demonstrate that HMGA2 is associated with replicating DNA. The ratio of HMGA2 to histone H3 was quantified in the input chromatin and the pulled-down fraction. The data revealed a 10- and 4-fold enrichment of HMGA2 at sites of ongoing replication in HT1080 and mESCs, respectively. Moreover, HMGA2 remained enriched (18- to 3-fold) at BrdU-labeled sites during HU treatment in both cell types (Figures S1C–S1F).

Taken together, these results indicated that HMGA2 is stably associated with replication foci containing ongoing and stalled replication forks. Furthermore, specifically in cells engaged in DNA replication, HMGA2 must be in close proximity to two key replication factors.

HMGA2 Protects against Nucleolytic Fork Collapse

In order to investigate whether the association of HMGA2 with replication sites or factories influences stalled fork stability, we first employed two recombinant fibrosarcoma cell lines, HT1080-C1 and HT1080-C2, which enabled us to substantially knock down endogenous HMGA2 levels via doxycycline-inducible expression of small hairpin RNA (shRNA) complementary to a sequence present in the 3′-untranslated region of HMGA2 mRNA (Figure 3A). As a control, parental HT1080 cells were first treated for 24 hr with increasing amounts of HU, and pulsed-field gel electrophoresis (PFGE) revealed that the fraction of shorter genomic DNA fragments (24–450 kb) increased with a concomitant decrease of larger fragments (450–2,200 kb) (Figure 3B). This shift in DNA fragmentation pattern is consistent with an overall increase in endonucleolytic fork collapse at elevated HU concentrations (Petermann et al., 2010).

HMGA2 knockdown (KD) in conjunction with HU treatment showed that HMGA2 suppressed fork collapse at low and high HU concentrations, as indicated by comparatively smaller amounts of shorter and larger amounts of longer genomic fragments when HMGA2 levels remained unperturbed (Figure 3C, left and middle panels). Control experiments with parental cells showed that this suppression was due to HMGA2 KD via shRNA expression (Figure 3C, right panels). Furthermore, no significant differences in apoptosis/necrosis or impact on cell cycle could be detected between HMGA2-expressing and KD cells after HU treatment (Figures S2A and S2B, and S2C and S2D, respectively). Neutral comet assays performed on HT1080 and HT1080-C1 cells corroborated our results obtained with PFGE (Figures S2E–S2I).

The specificity of shRNA-mediated HMGA2 KD was revealed in rescue experiments with expression vectors for HMGA2 transfected into HT1080-C1 cells after doxycycline-induced KD of the endogenous protein (Figures S3A and S3B). PFGE of genomic DNA isolated after 24 hr HU treatment and quantification of DNA fragments arising from double strand breaks (DSBs) in four independent experiments revealed that compared with control transfections, expression of exogenous HMGA2 significantly reduced fork collapse to a level comparable to that seen in the presence of endogenous HMGA2, i.e., in HU-treated cells in the absence of doxycycline (Figures S3C and S3D). HMGA2 KD in HT1080-C1 cells also confirmed the specificity of HMGA2 antibodies used in the colocalization and PLA studies shown in Figures 1 and 2 (also see Figures S3F and S3G, and S3H, respectively).

We next performed comet assays on pluripotent human ESCs after exposure to the DNA methylating agent methyl methanesulphonate (MMS), which also triggers fork arrest (Tercero and Diffley, 2001). We employed small interfering RNA (siRNA)-mediated HMGA2 KD and confirmed a general protective function exerted by endogenous HMGA2 against fork cleavage, as indicated by a significant increase in the amount of fragmented DNA seen in the comet tails when endogenous HMGA2 levels were reduced by about 50% (Figure 3D). Together, these results are in excellent agreement with previous findings that revealed significant protective effects of HMGA2 against drug-induced DNA damage in mouse embryonic fibroblasts and in human cancer cells expressing exogenous HMGA2 (Palmieri et al., 2011; Summer et al., 2009).

A general protective function of HMGA2 against nucleolytic fork collapse leading to DSBs was further substantiated by our finding that KD of endogenous HMGA2 in HT1080-C1/C2 cells led to a marked increase in the amount of the phosphorylated form of ataxia telangiectasia mutated (ATM-pSer1891) protein due to HU treatment (Figure 3E). We also found that HMGA2 does not affect the basal expression levels of ATM or ATR. In addition, the presence of HMGA2 showed no influence on ATR-mediated phosphorylation of CHK-1 (CHK1-pSer345) (Figure 3E), which serves as a cell signal indicating the presence of stalled replication forks (Cimprich and Cortez, 2008). The latter result also indicates that KD of endogenous HMGA2 does not impact the cell cycle during the period of HU treatment.

We next showed that both the intensity and number of foci formed by histone variant γ-H2AX at sites of either spontaneous or induced DNA breakage (Kinner et al., 2008) were significantly reduced in HMGA2-expressing human thyroid UTC8505 cancer cells before or immediately after MMS treatment (Figure S3I and S3J). Together, these results indicated that endogenous HMGA2 efficiently protects stalled forks against nucleolytic collapse in a variety of human cell types.

HMGA2 Protects Nascent DNA Strands and Promotes Replication Recovery

We next investigated whether HMGA2 protects the integrity of nascent DNA at arrested forks, using DNA fiber analyses. We found no significant effect on fiber length due to HMGA2 KD in untreated HT1080-C1/C2 cells. However, after exposure to HU for 24 hr, the length of prelabeled nascent DNA in HMGA2 KD cells was substantially reduced compared with that of cells with unperturbed HMGA2 levels (Figures 4A–4C). Control experiments using parental HT1080 cells confirmed that the instability of nascent DNA strands was due to HMGA2 KD via shRNA expression (Figure 4D).

In order to test whether the observed protective effect on nascent DNA strands in HMGA2-positive cells affects the restart of DNA synthesis upon release of the HU block, we performed BrdU incorporation assays. By determining the incorporation ratios in HMGA2-positive (i.e., doxycycline-untreated) and HMGA2 KD (i.e., doxycycline-treated) HT1080-C1/C2 cells during the 8 hr recovery after HU treatment, we observed that

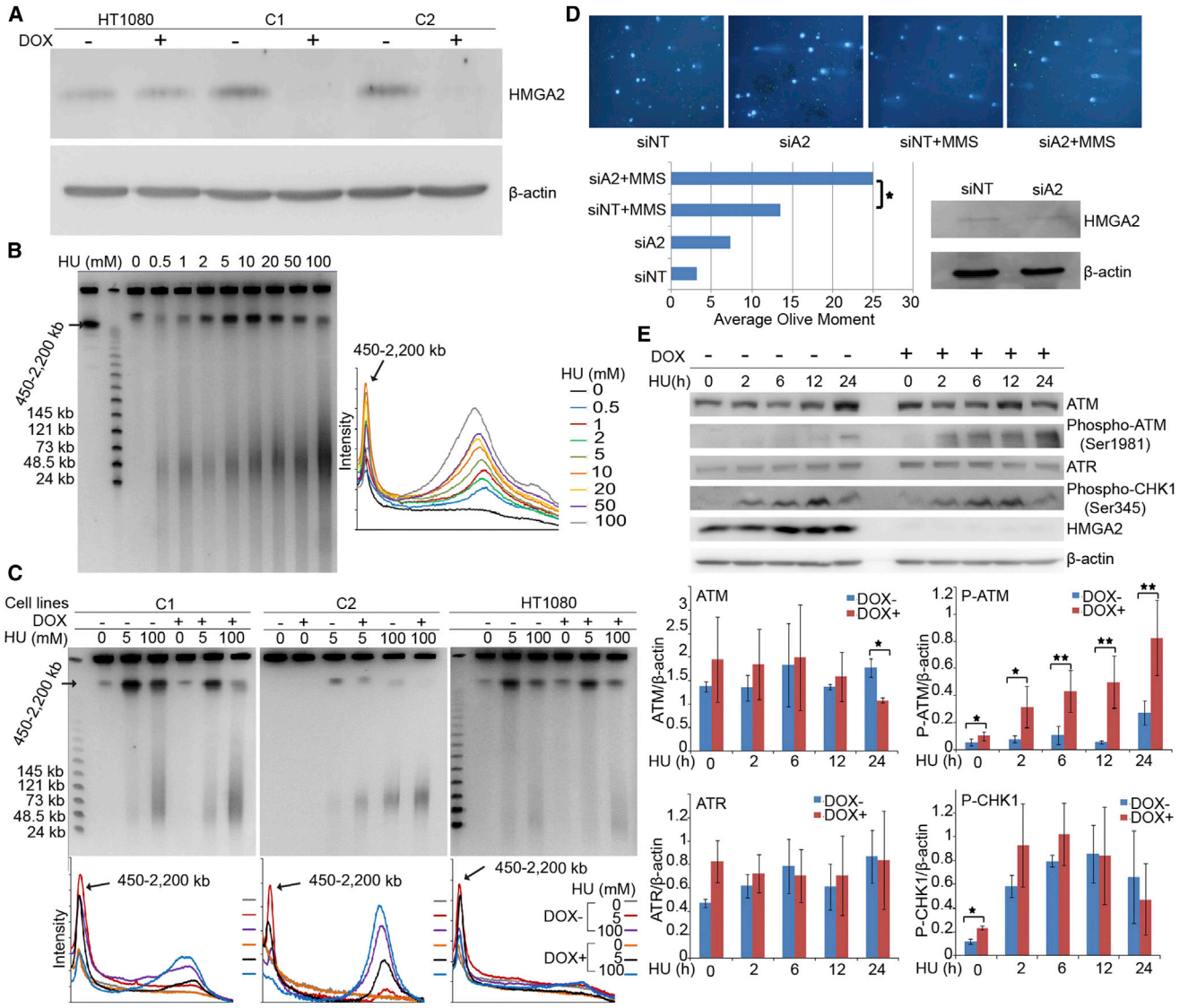

Figure 3. HMGA2 Protects Arrested Replication Forks from Collapsing into DSBs

(A) Western blot showing the downregulation of endogenous HMGA2 levels via doxycycline (DOX), which triggers HMGA2 shRNA expression and HMGA2 KD in two recombinant HT1080 cell lines. The parental cell line lacking the shRNA construct is shown as control.

(B) PFGE of genomic HT1080 DNA after treatment with increasing amounts of HU, as indicated. Intensity scans revealed a shift from larger to smaller fragments.

(C) Lack of HMGA2 led to the accumulation of smaller genomic fragments after HU treatment, indicating that more stalled forks collapsed as a result of endonucleolytic cleavage. Experiments were done in triplicate and one representative experiment is shown, with parental HT1080 cells as control. See also Figure S2.

(D) Comet assay performed on human ESCs (HUES7) after siRNA-mediated HMGA2 KD (western) and exposure to MMS without recovery. Nonspecific siRNA (siNT) was used as control. Experiments were done in triplicate. We show one representative example with 100 comets analyzed for each sample. Statistically significant differences in Olive moments are marked (*$p < 0.05$, **$p < 0.01$).

(E) Western blot analysis of ATM, phosphorylated ATM, ATR, HMGA2, and phosphorylated CHK1 using β-actin as the normalizing control before and after HU treatment, as indicated. The data presented here were obtained with HT1080-C1 cells. HT1080-C2 cells yielded very similar results. We show mean values of the respective protein ratios, SDs, and statistically significant differences from three independent experiments (*$p < 0.05$, **$p < 0.01$).

See also Figure S3.

the presence of HMGA2 ensured much shorter recovery times (Figure 4E). Taken together, these results indicated that HMGA2 does not affect nascent DNA integrity at normal ongoing replication forks, but helps to preserve these strands at stalled forks.

The shorter DNA synthesis recovery times in the presence of HMGA2 led us to investigate whether this coincided with changes in the amount of chromatin-bound replisome components. To that end, we performed quantitative analyses of total chromatin-associated PCNA and RPA (Görisch et al., 2008;

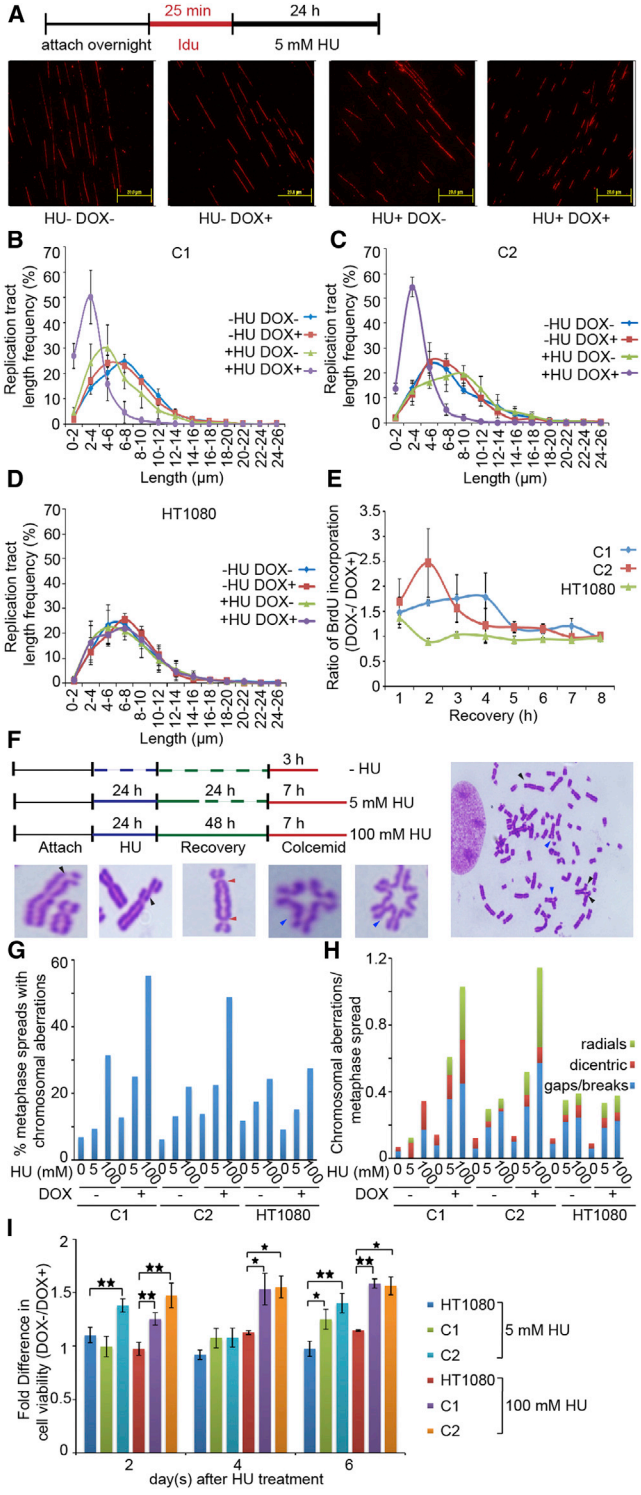

Figure 4. HMGA2 Preserves Nascent DNA Strand Integrity after HU Treatment and Promotes Genome Stability

(A–D) DNA fiber analyses after IdU pulse labeling and subsequent HU treatment. The principal experimental outline is diagrammed and representative examples of fibers under different experimental conditions are shown in (A). Mean values for replication tract length frequencies under various experimental conditions, as indicated, were determined for HT1080-C1 (B), HT1080-C2 (C), and parental HT1080 cells (D). Mean values and SDs are from three independent experiments.

(E) Replication forks recovered faster in the presence of HMGA2, as indicated by an increased ratio of BrdU incorporation in unperturbed C1 and C2 cells compared with KD conditions. No substantial differences due to doxycycline treatment were found for parental HT1080 cells. Mean values and SDs are from three independent experiments.

(F–H) HMGA2 promotes chromosomal stability after drug treatment. The experimental outline is diagrammed in (F). We show representative examples of different types (black arrowhead indicates gaps or breaks, red arrowhead indicates dicentric, blue arrowhead indicates radials) of chromosomal instabilities found to be increased due to lack of HMGA2. Experiments were performed twice. We show one as a representative example, including parental cells as control. For each sample, images of 40–60 spreads were taken and analyzed.

(I) MTT assays revealed that HMGA2 increased cell viability after HU treatment. Mean values of the respective ratios (DOX−/DOX+), SDs, and statistically significant differences in cell survival between cells with unperturbed and KD HMGA2 levels from three independent experiments are indicated (*$p < 0.05$, **$p < 0.01$).

See also Figure S4.

Yu et al., 2012). The data revealed that the amount of RPA and hyperphosphorylated RPA increased during HU treatment due to the uncoupling of DNA synthesis from unwinding at stalled forks, and that this increase was unaffected by HMGA2 KD (Figure S4). Furthermore, it is known that phosphorylation of RPA is cell-cycle dependent, and the lack of obvious phosphorylation pattern differences therefore corroborates our earlier data showing that KD of HMGA2 does not impact the cell cycle during HU treatment. We also showed that the amount of PCNA remained at comparable levels during HU treatment irrespective of HMGA2 KD (Figure S4). Interestingly, the amount of chromatin-bound HMGA2 increased substantially during HU treatment (Figure S4) and coincided with the onset of detectable genomic DNA fragmentation due to HU treatment (data not shown). We infer that HMGA2 at a global chromatin level neither interferes with the stability of a key replisome component, as indicated by similar amounts of chromatin-bound PCNA without HMGA2 KD, nor affects uncoupling of DNA synthesis from unwinding at arrested forks, as indicated by the comparable amounts of chromatin-bound RPA.

HMGA2 Promotes Genome Stability and Cell Survival after Replication Stress

The observed enhanced physical integrity of stalled forks mediated by HMGA2 could lead to a reduction in chromosomal aberrations (Schlacher et al., 2011, 2012). In order to test this possibility, we analyzed chromosome spreads at 24 or 48 hr after recovery from HU treatment. Strikingly, the results revealed that KD of HMGA2 significantly increased the percentage of metaphase spreads with aberrations (Figures 4F and 4G). In addition, the average number of HU-induced aberrations per metaphase spread increased in C1 and C2 cells when HMGA2 levels were reduced (Figure 4H). A closer inspection of the localization of small chromosomal deletions in cells with reduced HMGA2 levels revealed that more than 80% occurred on only one of the two sister chromatids, indicating that they were derived from replication problems (data not shown).

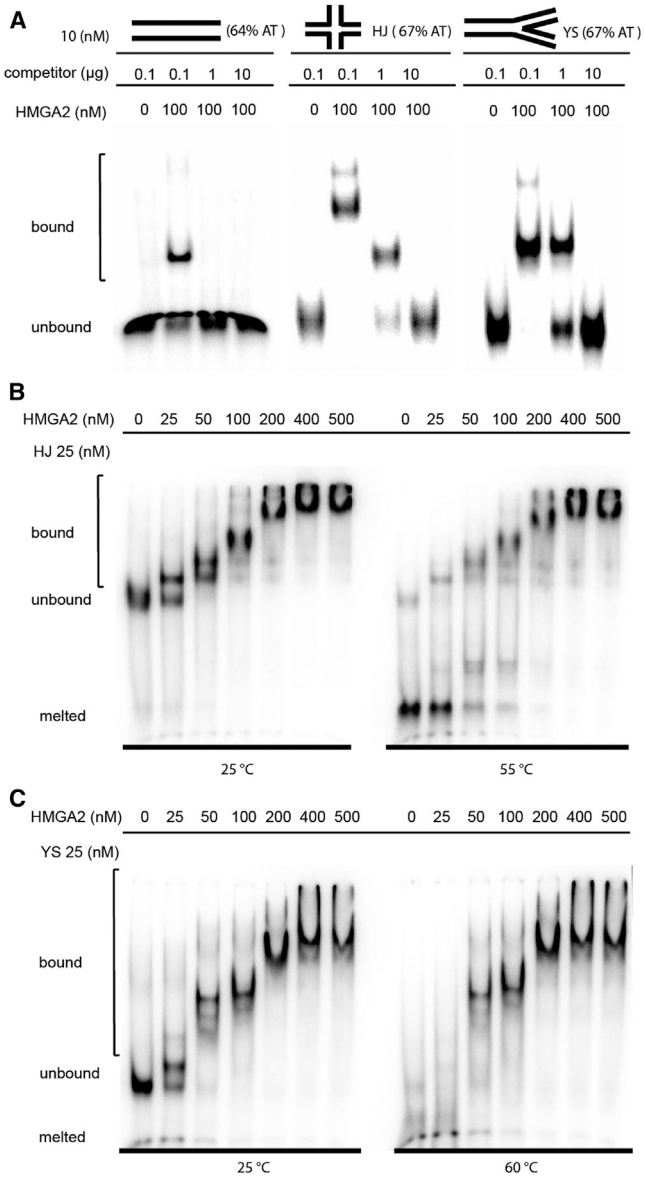

Figure 5. HMGA2 Preferentially Binds and Stabilizes Three- and Four-Way DNA Structures In Vitro

(A) EMSAs reveal higher affinities of HMGA2 for YSs and HJs compared with canonical duplex DNA in the presence of excess competitor DNA (salmon sperm DNA).

(B and C) HMGA2 binding protects HJs (B) and YSs (C) from temperature-induced duplex melting even at low protein:DNA stoichiometries.

See also Table S1.

Another potential consequence of HMGA2-mediated stalled fork protection is enhanced cell survival. We demonstrate that particularly at elevated HU concentrations, HMGA2 significantly promoted cell survival over a period of several days during recovery (Figure 4I). This is consistent with our previous data obtained with HMGA2-overexpressing A459 and HeLa cells (Summer et al., 2009), and with results from other studies (Natarajan et al., 2013; Palmieri et al., 2011). Furthermore, the rescue experiments described above also confirmed that the survival-promoting effect after HU challenge is specific for HMGA2 (Figure S3E).

HMGA2 Chaperones Three- and Four-Way DNA Junctions

To probe further into the role of HMGA2 in replication fork integrity at the molecular level, we studied interactions between HMGA2 and two branched DNA structures that are thought to be present at stalled forks (Branzei and Foiani, 2010; Petermann and Helleday, 2010; Ray Chaudhuri et al., 2012). We performed electrophoretic mobility shift assays (EMSAs) using in vitro assembled Y structures (YSs) and HJs. The results showed that in the presence of excess genomic competitor DNA, HMGA2 exhibited about 10-fold higher affinities for both branched DNAs in comparison with canonical AT-rich duplex DNA (Figure 5A; compare lanes with 1 μg competitor). Notably, this result is in agreement with previous reports that revealed a substantially higher affinity of the closely related HMGA1a protein for HJs (Hill et al., 1999; Hill and Reeves, 1997).

In order to test whether binding of HMGA2 enhanced the physical stability of YSs and HJs, we preincubated increasing amounts of HMGA2 with fixed amounts of DNA substrates and subsequently shifted the complexes to elevated temperatures. EMSAs revealed substantial protection for both branched DNA structures against DNA melting at HMGA2:DNA stoichiometries as low as 2:1 (Figures 5B and 5C).

HMGA2 Complements RecA in *E. coli* after Replication Stress

HMGA2 is a multifunctional protein in human cells. Its known pleiotropic effects are mediated by binding to chromatin via AT-hook domains and may involve protein-protein interactions via the C-terminal domain (Cleynen and Van de Ven, 2008; Fusco and Fedele, 2007; Pfannkuche et al., 2009). To provide further evidence for our hypothesis that HMGA2 is directly and solely responsible for the protection of stalled replication forks in vivo in the absence of other human factors, we chose heterologous *E. coli* as a model system in which the RecA protein primarily fulfills such a protective role (Courcelle and Hanawalt, 2003; Masai et al., 2010). We employed *E. coli recA* knockout (*ΔrecA*) and corresponding wild-type (WT) cells in conjunction with inducible, low-level expression of human HMGA2 (Figure S5A). Importantly, LexA-dependent reporter systems ensured that the expression of HMGA2 did not per se lead to activation of the *E. coli* SOS response in *ΔrecA* cells (data not shown).

We exposed *E. coli* cells to either HU or low pH (the latter treatment induces abasic DNA lesions, which in turn also trigger replication fork stalling; Maga et al., 2009). Colony-formation assays revealed that human HMGA2 fully restored *ΔrecA* cell viability to WT levels after exposure to HU, but showed no significant effect on WT cells (Figure 6A). Strikingly, HMGA2 also strongly protected against pH-induced cell death up to a level exceeding even WT cell viability (Figure 6B).

It has been shown that about 10% of *ΔrecA* cells are filamentous. Several lines of evidence indicate that this phenotype is due to a failure in chromosome segregation, which is caused by a lack of protection of stalled forks near so-called termination

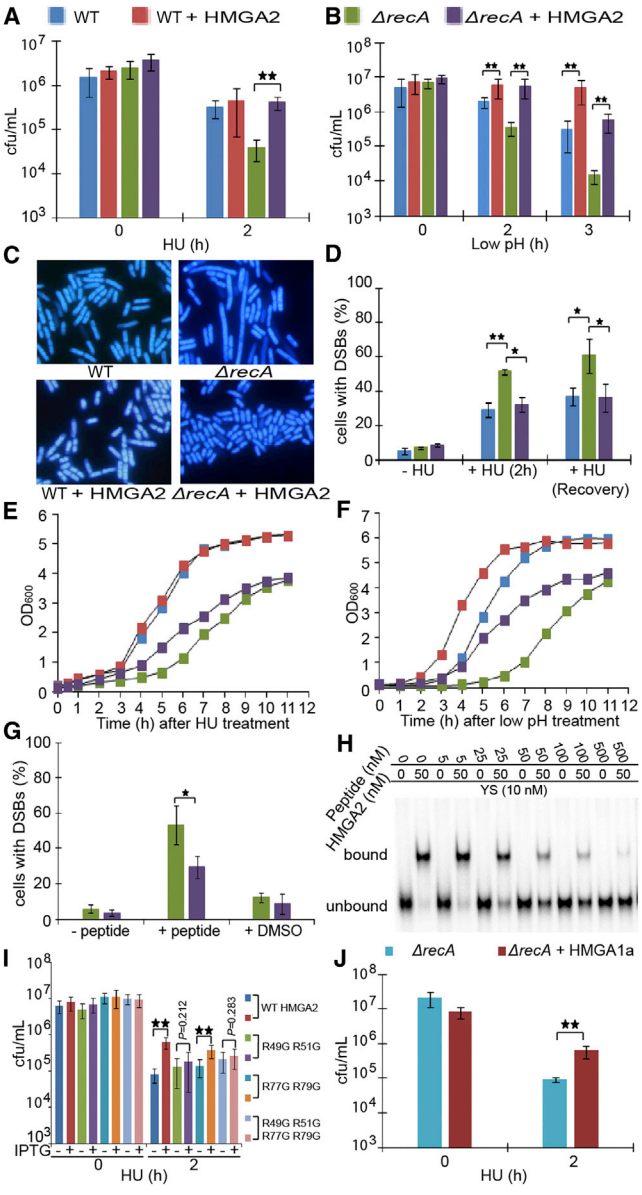

Figure 6. In Vivo RecA Complementation and Hexamer-Induced DNA Cleavage Assays

(A and B) Cell viability of WT and *ΔrecA* cells. (+) and (–) human HMGA2 expression, as indicated, was determined by colony-forming units (cfu) after exposure to either HU (A) or low pH (B). Mean cfu values, SDs, and statistically significant differences from three independent experiments are indicated (**$p < 0.01$). See also Figure S5A.

(C) DAPI staining of WT and *ΔrecA* cells, with and without HMGA2 expression, as indicated.

(D) Quantitation of comet assays performed either immediately after HU treatment or during a recovery period revealed significant protection against DSBs in the presence of RecA or HMGA2. SDs from three independent experiments and statistically significant differences are indicated (*$p < 0.05$, **$p < 0.01$). See also Figure S5B.

(E and F) HMGA2 expression promotes growth recovery after HU treatment in *ΔrecA* cells (E) and low pH treatment in WT and *ΔrecA* cells (F).

(G) Comet assays revealed a significant protection by HMGA2 against DSBs induced by the peptide wrwycr at branched DNA. Controls either lacked peptide or included DMSO as solvent. SDs from three independent experiments and statistically significant differences are indicated (*$p < 0.05$). See also Figure S5C.

(H) EMSAs performed after incubation of a fixed amount of HMGA2 with YSs at a 5:1 stoichiometry in the presence of increasing amounts of peptide revealed strong DNA binding competition.

(I and J) *ΔrecA* cells were transformed with various HMGA2 mutants carrying nonfunctional AT-hook(s) (I) or HMGA1a (J), and cell survival was determined by colony formation (cfu) before and after 2 hr of HU treatment with or without human protein induction as indicated. Mean values of cfu, SDs, and statistically significant differences from three independent experiments are indicated (**$p < 0.01$). See also Figures S5D–S5G.

(*ter*) DNA sites (Zyskind et al., 1992). Strikingly, we found that the presence of HMGA2 completely eliminated filamentous cell growth in *ΔrecA* cells (Figure 6C).

Impaired protection of arrested forks should also lead to an increase in DSBs in *ΔrecA* cells. We performed neutral comet assays and found that HMGA2, similarly to RecA in WT cells, significantly reduced the extent of HU-induced fork breakage, even after a recovery period (Figures 6D and S5B). This effect mirrors the protective function against nucleolytic lesions described above for HMGA2 in human cells.

In order to test whether fork protection mediated by HMGA2 results in faster fork recovery, as observed with human cells, we transferred WT and *ΔrecA* cells to new medium after 2 hr of HU treatment. Whereas HMGA2 expression had no effect on the growth of WT cells, *ΔrecA* cells recovered from replication arrest about 2 hr faster when HMGA2 was present (Figure 6E). Significant recovery improvements were also observed following low pH treatment, in particular for *ΔrecA* cells (Figure 6F).

HMGA2 Interferes with Peptide-Induced Cleavage of Branched DNA

Previous studies showed that the peptide hexamer wrwycr specifically binds to and blocks processing of YSs and HJs in bacterial cells (Gunderson and Segall, 2006; Kepple et al., 2008). This in turn leads to potent antimicrobial effects that are direct consequences of endonucleolytic attacks at these blocked, intermediate DNA structures, most likely at stalled forks.

We utilized the hexamer in combination with comet assays to test whether the presence of HMGA2 affects the extent of peptide-induced DNA cleavage in *ΔrecA* cells. The results revealed significantly fewer DSBs when HMGA2 was present (Figures 6G and S5C). Furthermore, EMSAs demonstrated strong binding competition between hexamer and HMGA2 on YSs (Figure 6H). Taken together, these results indicate that human HMGA2 alone is able to protect against DNA damage at stalled forks in *E. coli* via direct physical interactions with branched DNA.

Protection of Arrested Forks Requires Three Functional AT-hooks in *cis*

In order to provide further evidence for a direct association between HMGA2 and DNA at stalled forks as a prerequisite for its fork-stabilizing function, we inactivated individual AT-hooks via substitution of two critical residues per hook motif. Strikingly, HMGA2 variants carrying substitutions either in AT-hook 2 alone or in both AT-hooks 2 and 3 completely failed to complement RecA, while inactivation of AT-hook 3 alone led to a substantial (~4-fold) loss of protective power against HU-mediated cell

death compared with the parental protein (Figure 6I and S5D). In agreement with this finding, the suppression of filamentous cell growth of the *ΔrecA* strain by HMGA2 is also AT-hook dependent (Figure S5E). Hence, the presence of all three AT-hooks appears to be essential for an efficient replication fork-stabilizing function of HMGA2. Notably, previous results revealed that stable binding of HMGA1a to HJs in vitro also required the presence of three functional AT-hooks per HMGA1a molecule in *cis* (Hill et al., 1999).

We next confirmed these findings using human HT1080 cells and transient transfection assays with vectors for WT HMGA2 or AT-hook variants, followed by 24 hr of HU treatment and recovery. We first confirmed similar expression levels of recombinant HMGA2 (data not shown), and cell-survival assays revealed that expression of WT HMGA2, in addition to the endogenous protein, significantly increased cell viability compared with mock-transfected cells (Figure S5F). Complete loss of this protective effect was observed at 100 mM HU with HMGA2 variants harboring either an inactivated AT-hook 2 or inactivated AT-hooks 2 and 3 (Figure S5F). We were also able to confirm that three functional hooks are required to suppress fork collapse and promote cell survival in rescue experiments comparing expression vectors for WT and the HMGA2 variant harboring mutated hooks 2 and 3 (Figure S3E).

HMGA1a Complements RecA after Replication Stress

The AT-hook DNA-binding domains are highly conserved in the human HMGA1 and HMGA2 proteins. HMGA1, like HMGA2, is primarily expressed in pluripotent stem cells and most primary human cancer cells (Fusco and Fedele, 2007). Hence, we next determined whether the fork-stabilizing function of HMGA2 is conserved in the HMGA protein family. Specifically, we tested whether expression of the human HMGA1a variant in *E. coli ΔrecA* cells leads to increased viability after HU treatment. The results showed that fork protection appears to be an intrinsic and conserved function of human oncofetal HMGA proteins (Figures 6J and S5G).

HMGA2 Stabilizes Stalled Replication Forks in *S. cerevisiae*

In order to probe further into the mechanism of the proposed function of HMGA2 as an independent replication fork chaperone, we employed *S. cerevisiae* as a heterologous eukaryotic cell system that lacks HMGA orthologs. In yeast, the Mec1 protein (an ATR ortholog) has a crucial fork-stabilizing activity (Tercero et al., 2003). We therefore employed a *mec1Δsml1Δ* double-mutant strain carrying a galactose-inducible *GAL-HMGA2* gene construct stably integrated at the *TRP1* locus (Figure 7A) to test the extent to which human HMGA2 can complement this fork-stabilizing function during induced replication stress.

We first confirmed that the expression of human HMGA2 had no influence on the progression of the cell cycle in both WT and *mec1 Δsml1Δ* cells (data not shown). Mec1-deficient yeast cells are known to lose viability rapidly when treated with HU. We found that the expression of HMGA2 noticeably increased the viability of *mec1Δ sml1Δ* cells that were preexposed to HU (Figure 7B).

The *mec1Δ sml1Δ* cells then allowed us to directly probe into the structure of a synchronized, early-firing replicon on chromosome III (ARS305) after HU challenge in the presence or absence of HMGA2 (Lopes et al., 2001). The results of two-dimensional gel electrophoresis followed by Southern blotting revealed the presence of a so-called X-spike, which indicates regressed, chicken-foot-like fork structures in *mec1Δ sml1Δ* cells, but not in WT or *mec1Δ sml1Δ*-expressing HMGA2 cells, implying that HMGA2 partially protected stalled replication forks from collapsing into a X-spike (Figure 7C; Hu et al., 2012; Lopes et al., 2001). The fact that "late Y-forks" were seen for a longer period in *mec1Δ sml1Δ* cells expressing HMGA2 compared with *mec1Δ sml1Δ* cells is consistent with this notion (Figure 7C).

Since it is known that collapsed replication forks are prone to subsequent endonucleolytic attack leading to DSBs (Lopes et al., 2001; Petermann et al., 2010), we employed PFGE and observed a reduction of DNA fragmentation globally and on chromosome III in HU-treated *mec1Δ sml1Δ* cells when human HMGA2 was expressed (Figure 7D, top and bottom panels, respectively). Hence, the observed increase in viability of HMGA2-positive *mec1Δ sml1Δ* cells coincides with a substantial protection against endonucleolytic cleavage of DNA, which in turn appears to be due, at least in part, to the prevention of fork regression/collapse.

DISCUSSION

A fraction of replication forks always encounter obstacles during translocation along the parental DNA, and physical stabilization of arrested forks represents a first line of defense against fork collapse (Branzei and Foiani, 2010; Mirkin and Mirkin, 2007). Stabilization can be achieved by the fork protection complex or is generally initiated by recognition of ssDNA (Branzei and Foiani, 2010; Courcelle et al., 2003; Errico and Costanzo, 2010, 2012; Schlacher et al., 2011, 2012). We show in this study that HMGA2 specifically equips ESCs and cancer cells with an additional fork protection mechanism, which differs from other pathways (Atkinson and McGlynn, 2009; Courcelle and Hanawalt, 2003; Petermann and Helleday, 2010; Schlacher et al., 2011, 2012).

Our results revealed stable association of HMGA2 with human replication foci containing either ongoing or arrested forks. It has been described that the highly homologous HMGA1a protein interacts with subunits of the human origin recognition complex (ORC) via its C-terminal domain (Thomae et al., 2008, 2011). Given the high degree of conservation, it is possible that HMGA2 also interacts with ORC and remains there after initiation of replication has occurred (Natsume and Tanaka, 2010).

We have shown that the presence of HMGA2 does not impact ongoing replication. In addition, the lack of *HMGA* genes does not alter cell growth, indicating that HMGA proteins are not essential factors for DNA replication (Beitzel and Bushman, 2003; Palmieri et al., 2011). However, when forks are arrested, the presence of HMGA2 protects forks from nucleolytic collapse.

Our biochemical data revealed that HMGA2 binds with high affinity to branched DNA. Current models favor two types of branched structures forming at stalled forks (Branzei and Foiani, 2010): the three-way junction, which resembles an unperturbed

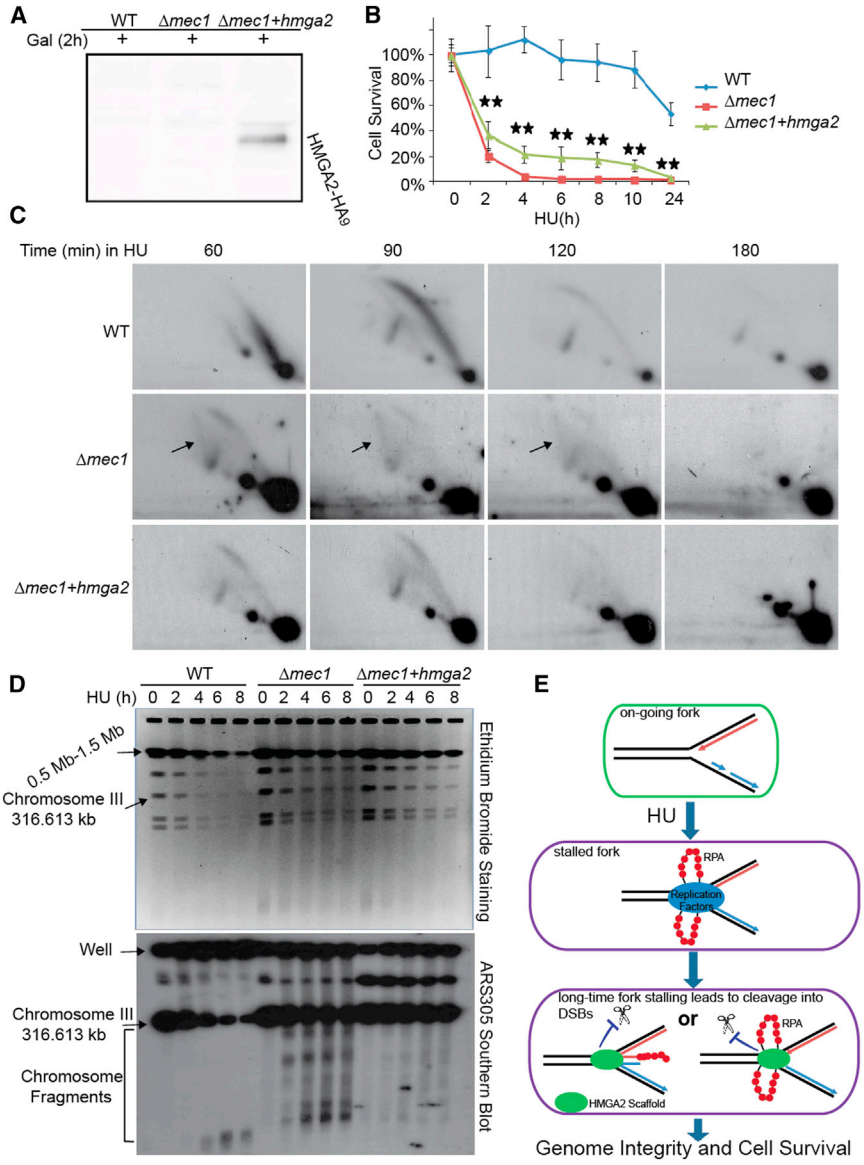

Figure 7. HMGA2 Stabilizes Stalled Replication Forks in *S. cerevisiae*

(A) Western blot showing expression of HMGA2-$(HA)_9$ after 2 hr galactose induction in WT, *Δmec1*, and *Δmec1+HMGA2* strains.

(B) Cell viability was determined as cfu after exposure to 30 mg/ml HU for the times indicated. Mean values of cfu, SDs, and statistically significant differences from three independent experiments are indicated (**$p < 0.01$).

(C) Replication intermediates arising from the *ARS305* origin in response to HU were studied by two-dimensional gel electrophoresis. The position of the X-spike signal that contains reversed, chicken-foot-like forks is indicated by black arrows. Experiments were done in duplicate and one representative experiment is shown.

(D) PFGE analysis of the occurrence of DSBs in the yeast genome, as revealed by ethidium bromide staining (top), and in chromosome III (probe: *ARS305*; bottom) during HU treatment.

(E) Model of HMGA2-mediated protection of stalled replication forks. See text for details.

YS, and the HJ or chicken-foot structure that results from fork reversal. Here, we propose that HMGA2 forms a protective scaffold with branched DNA at arrested forks. This interaction might be favored in human cells by a high local concentration of HMGA2 inside replication foci, perhaps through interactions with ORC or replisome components, such as Ku70/80 (Sgarra et al., 2008). The proposed HMGA2 scaffold physically stabilizes stalled fork structures, seemingly without interfering with PCNA association or RPA loading, thereby promoting genome integrity and cell survival.

In this context, it is possible that the presence of HMGA2 at stalled replication forks engages its recently discovered dRP/AP lyase activity to more efficiently initiate base excision repair at abasic sites (Summer et al., 2009). The increased cell viability in the presence of HMGA2 observed in the heterologous *E. coli* system after low pH challenge already points to such a possibility. Further studies are needed to shed more light on this emerging HMGA2-mediated connection between fork stability and DNA repair.

Our model suggests that HMGA2 can work independently of other human factors in stabilizing stalled forks. This is based on results obtained with two heterologous cell systems. In *E. coli*, complementation of RecA led to a reduction in DNA strand breaks, the elimination of filamentous growth, and an increase in cell survival and recovery times after replication stress. Furthermore, the *E. coli* system provided evidence that HMGA2 works directly at the level of replication forks. First, by employing the hexapeptide wrwycr, which specifically binds to branched DNA structures in vivo and triggers DNA strand breaks at stalled forks and HJs (Gunderson and Segall, 2006), we demonstrated that HMGA2 suppresses peptide-induced DNA lesions most likely via binding competition. Second, variants of HMGA2 carrying inactivated AT-hook domains failed to complement RecA, hence revealing a requirement for the presence of all three AT-hooks for fork protection. This scenario is supported by the finding that stable binding of HMGA1a to HJs also requires the presence of three functional AT-hooks (Hill et al., 1999).

The yeast system allowed us to demonstrate that the fork-stabilizing function of HMGA2 reduced, to a discernible extent, the number of pathological forks. This contributed to an overall reduction in genome fragmentation and an increase in cell viability after HU-induced replication stress. The yeast Mec1/ATR knockout system in conjunction with western blotting of human ATR/pCHK1 proteins also indicated that HMGA2 functions

independently of the fork-based ATR-pCHK1 signaling pathway. Notably, the dynamics of phosphorylation at serine 345 of CHK1 were not affected by HMGA2 protein levels. This is in agreement with recent data that hinted at a CHK1-independent function of HMGA2 in preventing apoptosis as a result of replication stress (Natarajan et al., 2013).

The cellular consequences of replication stress depend on the extent of the DNA damage inflicted and the genetic background. We exposed cells to high HU concentrations for 24 hr, which led to a significant increase in chromosomal aberrations and a decrease in cell viability. The fact that HMGA2 efficiently protects stalled forks under these conditions implies that the proposed interaction must be quite stable—a scenario that is strongly supported by our biochemical data. Although HMGA2 seemingly acts on its own when all three AT-hooks are functional, contributing protein-protein interactions between HMGA2 and other human factors (e.g., Ku70/80) are not ruled out (Sgarra et al., 2008).

Our data show that arrested fork stabilization by HMGA2 leads to significantly shorter replication recovery times. The fact that HMGA2 protects nascent DNA strands might indicate that stalled forks are maintained in a functional, replisome-bound state. However, our combined data indicate that it is more likely that forks are rescued by the firing of dormant replication origins located within the same replicon (Blow et al., 2011; Ge and Blow, 2010; Kawabata et al., 2011) or perhaps through recruitment of PrimPol (Mourón et al., 2013).

HMGA proteins are primarily expressed in pluripotent stem cells and in most human malignancies (Cleynen and Van de Ven, 2008; Fedele and Fusco, 2010; Fedele et al., 2010). These cells are highly metabolically active and exhibit fast DNA replication cycles. Therefore, it is conceivable that stalling of replication forks occurs quite frequently in these cell types. We employed a variety of ESC and cancer cell lines to demonstrate that HMGA2 has a potent general fork chaperone function. For ESCs, this protection pathway may be an important component of a genome surveillance system to prevent mutations and aberrations. For cancer (stem) cells, it provides a highly effective first-line defense mechanism against DNA-targeting chemotherapeutic agents currently used in the clinic. Given that HMGA2 is not translated in normal somatic cells, this HMGA2 fork chaperone function provides a promising rationale for the development of therapeutic strategies to specifically target cancer (stem) cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Chemicals

Recombinant HT1080-C1 and HT1080-C2 cell lines were generated from individual colonies after lentiviral transduction of HT1080 cells with pTRIPz-shHMGA2 (Origene), followed by puromycin selection. HMGA2-expressing and mock UTC8505 transfectants were generated as previously described (Summer et al., 2009). Culture conditions for HUES7 were described previously (Tan et al., 2007). mESCs J1 were cultured in standard ESC media. Cancer cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen). HMGA2 was downregulated by induction of shHMGA2 (GCCAACTCTTCTATTTATGGAT) with 2 μg/ml doxycycline hyclate (Sigma), which was applied once every day for 4 days.

Antibodies

The primary antibodies used were rabbit polyclonal anti-HMGA2 (1:100; Abcam), rabbit monoclonal anti-HMGA2 (Cell Signaling), rabbit monoclonal anti-ATM (1:1,000; Cell Signaling), rabbit polyclonal anti-ATR (1:1,000; Cell Signaling), mouse monoclonal anti-Phospho-ATM(Ser1981) (1:1,000; Cell Signaling), rabbit polyclonal anti-Phospho-CHK1(Ser345) (1:1,000; Cell Signaling), mouse monoclonal anti-PCNA (1:2,000; Cell Signaling), rabbit polyclonal anti-PCNA (1:100; Abcam), mouse monoclonal anti-RPA32 (1:100; Abcam), mouse monoclonal anti-BrdU (1:100, B44; BD) to detect IdU, and rat monoclonal anti-BrdU (1:100, BU1/75; abcam). The secondary antibodies were donkey anti-rabbit immunoglobulin G Alexa Fluor 488 (1:200; Invitrogen), goat polyclonal anti-mouse TRITC (1:100; Abcam), and horseradish peroxidase-conjugate goat anti-rabbit (1:1,000; Santa Cruz).

In Situ PLA

In situ PLA was performed using the Duolink Detection Kit (Olink Bioscience) with PLA PLUS and MINUS probes for rabbit and mouse based on the manufacturer's protocol. Briefly, HU-nontreated HT1080 cells grown on glass coverslips were labeled with 10 μM EdU for 15 min followed by immediate fixation and further staining. HU-treated cells were labeled with 10 μM EdU for 15 min before 5 mM HU was added into the same medium for 16 hr. Cells were washed once with PBS and fixed in 4% formaldehyde in PBS (pH 7.4) for 10 min. After three washes with PBS buffer, the cells were permeabilized with either 0.5% Triton in pH 7.4 PBS for 10 min or ice-cold methanol at −20°C for 10 min when PCNA antibodies were applied. Cells were washed with PBS three times before blocking with 5% BSA in PBS with Tween-20 (PBST) buffer for 1 hr. The cells were incubated with different combinations of primary antibodies (rabbit monoclonal anti-HMGA2 [1:400; Cell Signaling], mouse monoclonal anti-RPA32 [1:100; Abcam], mouse monoclonal anti-PCNA [1:100; Cell Signaling], and rabbit polyclonal anti-PCNA [1:100; Abcam]) diluted in PBST with 1% BSA in a 37°C water bath for 1 hr. After three washes with PBST, the cells were incubated with oligonucleotide-conjugated probe secondary antibodies. The recognition of primary antibodies initiated a DNA-amplification-based reporter system that generated a signal only when the distance between two proteins was <40 nm. The labeled EdU was detected with the use of a Click-iT EdU Imaging Kit (Invitrogen). Cells were counterstained with DAPI and mounted. Images were obtained with a Zeiss LSM 710 confocal microscope.

E. coli Cell-Survival Assays

Overnight cultures were inoculated (1:200) into fresh lysogeny broth (LB) medium with or without isopropyl β-D-1-thiogalactopyranoside and continued to culture for about 2 hr until OD_{600} reached 0.3–0.4. For low-pH treatment, equal numbers of cells were resuspended in 3 ml LB medium (pH 3.25) and incubated at 37°C for 2 hr without shaking. For HU treatment, equal numbers of cells were resuspended in 2.7 ml fresh LB with or without 300 mM HU and incubated at 37°C for 2 hr at 100 rpm. Equal numbers of cells were plated in serial dilutions. Assays were performed in triplicate for each serial dilution.

Full details regarding other experimental procedures can be found in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.01.014.

AUTHOR CONTRIBUTIONS

H.Y., H.H.L., N.T., T.K., S.D.G., U.S., and P.D. designed the experiments. H.Y., H.H.L., N.T., P.S., T.C., and S.N. performed the experiments. H.Y., P.S., and S.N. performed statistical analyses. P.D. and H.Y. wrote the manuscript.

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